Technetium Complexes of a Hydrazinonicotinamide-Conjugated Cyclic Peptide and 2-Hydrazinopyridine: Synthesis and Characterization

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Received August 13, 1998

Ternary ligand technetium complexes of a hydrazinonicotinamide-conjugated cyclic peptide (HYNICtide: cyclo-(D-Val-NMeArg-Gly-Asp-Mamb(5-(6-(6-hydrazinonicotinamido)hexanamide)))) and 2-hydrazinopyridine (HYPY) were prepared and characterized by various spectroscopic methods. The HPLC concordance experiments for ^{99m}Tc and ^{99}Tc analogues show clearly that the same complexes are prepared on the no-carrier-added (^{99m}Tc) and the carrier-added (^{99}Tc) levels. Using a chirality experiment, it was demonstrated that the presence of two radiometric peaks in the HPLC chromatograms of RP444, RP445, and RP446 is due to the resolution of diastereomers, which result from the presence of chiral cyclic peptide and the formation of two enantiomers of the technetium chelate. In a ligand challenge experiment, we found that the high solution stability of these ternary ligand [^{99m}Tc]HYNICtide complexes is due to their kinetic inertness. The 1:1:1:1 composition for Tc:HYNICtide:L:tricine (L = TPPTS, TPPDS, and TPPMS) in these ternary ligand [^{99}Tc]HYNICtide complexes is confirmed by ^{1}H NMR and FAB mass spectral data and is completely consistent with that determined on the tracer (^{99m}Tc) level. In addition, the IC_{50} values of RP444, RP445, and RP446 and the two isomeric forms of RP444 were determined using a platelet IIb/IIIa binding assay. Both isomeric forms of RP444 were found to have the same binding affinity (IC_{50} = 13 ± 2 nM). Complexes [^{99}Tc(HYPY)(PPh_3)$_2$$Cl_2$] and [^{99}Tc(HYPY)($PPh_3$)(tricine)] were isolated from the reaction of HYPY with [*n*-Bu_4N][$TcOCl_4^-$] in the presence of excess tricine and triphenylphosphine. [^{99}Tc(HYPY)(PPh_3)-(tricine)] serves as a model for ternary ligand [^{99m}Tc]HYNICtide complexes. Both complexes have been characterized by HPLC, spectroscopic (IR, NMR, and FAB-MS) methods, and elemental analysis. The HPLC concordance for complexes [^{99m}Tc(HYPY)(PPh_3)(tricine)] and [^{99}Tc(HYPY)(PPh_3)(tricine)] shows that the two complexes are identical. The NMR (^{1}H and ^{13}C) data suggests that the complex [^{99}Tc(HYPY)(PPh_3)(tricine)] have an octahedral coordination geometry with a monodentate diazenido HYPY, a tetradentate tricine, and a monodentate triphenylphosphine coligand.

Introduction

There is currently considerable interest in labeling small peptides with ^{99m}Tc for the development of target specific imaging agents.[1−4] We are interested in the ^{99m}Tc-labeling of glycoprotein IIb/IIIa (GPIIb/IIIa) receptor antagonists for the development of thrombosis imaging agents.[5−9] Recently we labeled a hydrazinonicotinamide (HYNIC) derivatized cyclic peptide (HYNICtide: cyclo(D-Val-NMeArg-Gly-Asp-Mamb-(5-(6-(6-hydrazinonicotinamido)hexanamide)))) using tricine and a water soluble phosphine (Chart 1: TPPTS, trisodium triphenylphosphine-3,3′,3″-trisulfonate; TPPDS, disodium triphenylphosphine-3,3′-disulfonate; TPPMS, sodium triphenylphosphine-3-monosulfonate) as coligands.[8] The combination of HYNICtide with tricine and phosphine produces a new ternary ligand system which forms stable technetium complexes, [^{99m}Tc(HYNICtide)(tricine)(L)] (RP444, L = TPPTS; RP445, L = TPPDS; RP446, L = TPPMS) in high yield and high specific activity (≥20000 Ci/mmol). It was found that these ternary ligand [^{99m}Tc]HYNICtide complexes are formed as equal mixtures of two isomeric forms and are stable for ≥6 h in the reaction mixture and in dilute solution. The composition of these complexes was determined to be 1:1:1:1 for Tc:HYNICtide:L:tricine (L = TPPTS, TPPDS, and TPPMS) through a series of mixed-ligand experiments on the tracer (^{99m}Tc) level. In the canine arteriovenous (AV) and deep vein thrombosis (DVT) models, RP444, RP445, and RP446 have been shown to be able to detect rapidly growing arterial and venous thrombi. RP444 is under clinical evaluation as a new thrombus agent.

To understand the coordination chemistry of this unique ternary ligand system, we prepared the ^{99}Tc analogues of RP444,

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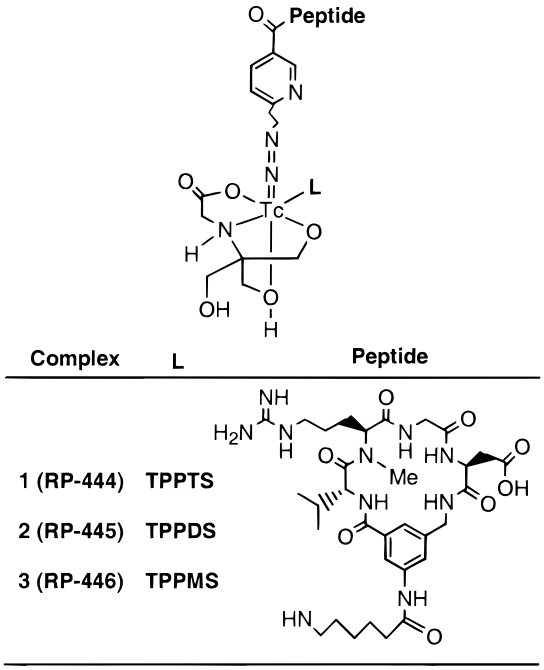
(1) Liu, S.; Edwards, D. S.; Barrett, J. A. *Bioconjugate Chem.* **1997**, *8*, 621.
(2) Edwards, D. S.; Liu, S. *Transition Met. Chem.* **1997**, *22*, 425.
(3) Lister-James, J.; Moyer, B. R.; Dean, R. T. *Q. J. Nucl. Med.* **1997**, *41*, 111.
(4) Lister-James, J.; Moyer, B. R.; Dean, R. T. *Q. J. Nucl. Med.* **1996**,*40*, 221.
(5) Liu, S.; Edwards, D. S.; Looby, R. J.; Harris, A. R.; Poirier, M. J.; Barrett, J. A.; Heminway, S. J.; Carroll, T. R. *Bioconjugate Chem.* **1996**, *7*, 63.
(6) Liu, S.; Edwards, D. S.; Looby, R. J.; Harris, A. R.; Poirier, M. J.; Rajopadhye, M.; Bourque, J. P. *Bioconjugate Chem.* **1996**, *7*, 196.
(7) Barrett, J. A.; Damphousse, D. J.; Heminway, S. J.; Liu, S.; Edwards, D. S.; Looby, R. J.; Carroll, T. R. *Bioconjugate Chem.* **1996**, *7*, 203.
(8) Edwards, D. S.; Liu, S.; Harris, A. R.; Looby, R. J.; Ziegler, M. C.; Heminway, S. J.; Barrett, J. A.; Carroll, T. R. *Bioconjugate Chem.* **1997**, *8*, 146.
(9) Barrett, J. A.; Crocker, A. C.; Damphousse, D. J.; Heminway, S. J.; Liu, S.; Edwards, D. S.; Harris, A. R.; Looby, R. J.; Lazewatsky, J. L.; Kagan, M.; Mazaika, T. J.; Carroll, T. R. *Bioconjuate Chem.* **1997**, *8*, 155.

10.1021/ic980973o CCC: $18.00
Published on Web 03/04/1999

Chart 1. Ternary Ligand Technetium Complexes



RP445, and RP446. We also used 2-hydrazinopyridine (HYPY) as the model for the HYNICtide and triphenylphosphine (PPh_3) for TPPTS to prepare the ternary ligand complex [Tc(HYPY)-(PPh_3)(tricine)]. In this report, we present synthesis, HPLC, and spectroscopic characterization of technetium complexes of the HYNICtide and HYPY.

Experimental Section

Materials. TPPTS (trisodium triphenylphosphine-3,3′,3″-trisulfonate) was purchased from Aldrich Chemical Co. and was purified according to the literature method.[10] TPPDS (dipotassium triphenylphosphine-3,3′-disulfonate) was purchased from STREM Chemicals Co., Newburyport, MA, and was used as received. TPPMS (sodium triphenylphosphinebenzene-3-monosulfonate) was purchased from TCI America, Portland, OR, and was used without further purification. [*n*-Bu_4N]-[$^{99}TcOCl_4$] was prepared according to Davison's procedure.[11] $Na^{99m}TcO_4$ was obtained from a Technelite $^{99}Mo/^{99m}Tc$ generator, DuPont Merck Pharmaceutical Co., North Billerica, MA. *N*-benzyl-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-BA) and *N*-((*R*)-(+)-α-methylbenzyl)-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-MBA) were prepared and purified according to the procedure reported previously[12] and recrystallized twice from methanol before use for radiolabeling. The synthesis and biological properties of HYNICtide have been reported previously.[13]

Instruments and Methods. Proton NMR spectra were recorded on a 270 MHz Bruker spectrometer or a Bruker DRX 600 MHz spectrometer. ^1H NMR data were reported as δ in ppm relative to TMS: δ (multiplicity, number of hydrogen nuclei, assignment, coupling constant). FAB-MS spectra were obtained using a Katos Concept II H32Q instrument (Cs^+-LSIMS with positive ion detection) and a mixture of thioglycerol and water as the matrix. Infrared spectra were recorded as KBr disks in the range 4000−400 cm^{-1} on a Nicolet 5DXB IR spectrometer. Elemental analyses were performed by Galbraith Laboratories, INC., Knoxville, TN. The high-performance liquid chromatography (HPLC) methods used a Hewlett-Packard Model 1050 or Model 1090 instrument equipped with both a radiometric NaI detector and a Rainin Dynamax UV detector (Model UV-C, λ = 340 nm).

HPLC method 1 used a Vydac C_{18} column (4.6 mm × 250 mm, 300 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase from 100% A (10 mM phosphate buffer, pH 6) to 30% B (acetonitrile) at 15 min and 75% B at 25 min.

HPLC method 2 used a heated (50 °C) Cosmosil C_{18} column (4.6 mm × 250 mm, 500 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase from 92% A (0.025 M succinate buffer, pH 5) and 8% B (acetonitrile) to 88% A and 12% B at 20 min. The mobile phase was isocratic at retention times 20−30 min with 88% A and 12% B, and 31−40 min with 50% A and 50% B.

HPLC method 3 used a heated (50 °C) Zorbax C_{18} column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min. The mobile phase was isocratic from 0 to 20 min using 100% mobile phase A (87:13 (0.025 M phosphate buffer, pH 8):acetonitrile), and from 21 to 30 min using 100% mobile phase B (50:50 (0.025 M phosphate buffer, pH 8):acetonitrile).

HPLC method 4 used a Zorbax C_{18} column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min. The mobile phase was isocratic from 0 to 45 min using 81% A (0.01 M phosphate buffer, pH 6) and 19% B (acetonitrile), and from 46 to 55 min using 50% A and 50% B.

HPLC method 5 used a Zorbax C_{18} column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase from 90% A (0.01 M phosphate buffer, pH 6) and 10% B (acetonitrile) to 75% A and 25% B at 20 min, and to 25% A and 75% B at 25 min.

HPLC method 6 used a Zorbax C_{18} column (4.6 mm × 250 mm, 80 Å pore size) at a flow rate of 1 mL/min with a gradient mobile phase from 95% A (0.01 M phosphate buffer, pH 6) and 5% B (acetonitrile) to 90% A and 10% B at 30 min. The mobile phase was isocratic at 31−40 min with 50% B.

Synthesis of ^{99}Tc Complexes. ***CAUTION!*** ^{99}Tc is a low-energy (0.292 MeV) β^- emitter with a half-life of 2.12×10^5 years. All manipulations of solutions and solids were performed in a laboratory approved for the handling of low-level radioisotopes, and normal safety procedures must be used at all times to prevent contamination.

[^{99}Tc]RP444 from [*n*-Bu_4N][$^{99}TcOCl_4$]. To a 10 mL vial was added tricine (215 mg, 1.2 mmol) in H_2O (1 mL), followed by [*n*-Bu_4N]-[$^{99}TcOCl_4$] (4.0 mg, 0.008 mmol) in methanol (1 mL), HYNICtide·2TFA (10.5 mg, 0.01 mmol), and TPPTS (15 mg, 0.026 mmol) in H_2O (2 mL). The solution was heated at 100 °C for 30 min and was then allowed to cool to room temperature. The product, [^{99}Tc]RP444, was separated from the reaction mixture by HPLC (method 1). The product fraction at retention time from 10 to 11 min was collected. The collections were combined, and the solvent was removed under reduced pressure. The residue was dissolved in water (1.0 mL) and was then repurified (method 2) by collecting the fraction at retention time from 22 to 26 min. [^{99}Tc]RP444 was desalted using water and acetonitrile as the mobile phase.

[^{99}Tc]RP444 from [NH_4][$^{99}TcO_4$]. To a 10 mL vial were added tricine (100 mg, 0.56 mmol), HYNICtide·2TFA (14 mg, 0.013 mmol), TPPTS (28 mg, 0.049 mmol), [NH_4][$^{99}TcO_4$] (1.8 mg, 0.01 mmol) in water (0.5 mL), and 2.5 mL of 0.025 M succinate buffer, pH = 5. The mixture was heated at 100 °C for 20 min. After cooling to room temperature, the product, [^{99}Tc]RP444, was separated from the reaction mixture by HPLC (method 1). [^{99}Tc]RP444 was shown by HPLC to be the same as that prepared from [*n*-Bu_4N][$^{99}TcOCl_4$].

[^{99}Tc]RP445. To a suspension of tricine (160 mg, 0.90 mmol), HYNICtide·2TFA (10.5 mg, 0.01 mmol), and TPPDS (30 mg, 0.064 mmol) in H_2O (2 mL) was added [*n*-Bu_4N][$^{99}TcOCl_4$] (2.5 mg, 0.005 mmol) in methanol (1 mL). The reaction mixture was heated at 100 °C for 15 min and was then allowed to cool to room temperature for HPLC purification. The product, [^{99}Tc]RP445**,** was separated from the reaction mixture by HPLC (method 1). The product fraction at retention time 13−15 min was collected. The collections were combined and the solvent was removed under reduced pressure. The residue was dissoved in water (1.0 mL) and was repurified by HPLC (method 4).

(10) Kuntz, E. U.S. Patent 4,248,802, 1981.
(11) Davison, A.; Trop, H. S.; DePamphilis, B. V.; Jones, A. J. *Inorg. Synth.* **1981**, *21*, 160.
(12) Liu, S.; Edwards, D. S.; Harris, A. R. *Bioconjugate Chem.* **1998**, *9*, 583.
(13) Rajopadhye, M.; Harris, T. D.; Yu, K.; Glowacka, D.; Damphousse, P. R.; Barrett, J. A.; Heminway, S. J.; Edwards, D. S.; Carroll, T. R. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 955.

[^{99}Tc]RP445 was desalted using method 1 and was found to be free from uncoordinated HYNICtide.

[^{99}Tc]RP446. To a suspension of tricine (130 mg, 0.73 mmol) and HYNICtide·2TFA (16 mg, 0.015 mmol), and TPPMS (20 mg, 0.055 mmol) in H_2O (2 mL) was added [*n*-Bu_4N][$^{99}TcOCl_4$] (5.0 mg, 0.01 mmol) in methanol (1 mL). The reaction mixture was heated at 100 °C for 20 min and was then allowed to cool to room temperature for HPLC purification. The product, [^{99}Tc]RP446**,** was separated from the reaction mixture by HPLC (method 5). The product fraction at retention time 18−20 min was collected. The collections were combined and the volatiles were removed under reduced pressure. The residue was dissoved in water (1.0 mL) and was repurified by HPLC (method 5). [^{99}Tc]RP446 was desalted using water and acetonitrile as the mobile phase.

[^{99}Tc(HYPY)(PPh_3)$_2$$Cl_2$]. To a solution of [*n*-Bu_4N][$TcOCl_4$] (380 mg, 0.76 mmol) in chloroform (10 mL) was added tricine (720 mg, 4.0 mmol) and HYPY·2Cl (350 mg, 1.92 mmol) in a mixture of water (10 mL) and methanol (15 mL), and triphenylphosphine (630 mg, 2.4 mmol) in chloroform (10 mL). The solution became dark red immediately after addition of HYPY·2Cl. The reaction mixture was refluxed for 50−60 min while a red solid formed. After cooling to room temperature, the solid was filtered, washed with methanol and diethyl ether, and dried under vacuum. The yield was 430 mg. Anal. Calcd (found) for $C_{36}H_{34}Cl_2N_3P_2Tc$: C, 61.47 (61.84); H, 4.28 (4.22); N, 5.25 (5.47). LSIMS: m/z = 800 for M + H ($[C_{41}H_{35}Cl_2N_3P_2Tc]^+$), 764 for M − Cl ($[C_{41}H_{34}ClN_3P_2Tc]^+$), 537 for M − PPh_3 ($[C_{23}H_{19}Cl_2N_3PTc]^+$), and 503 for M − Cl − PPh_3 ($[C_{23}H_{19}ClN_3PTc]^+$). ^{1}H NMR ($CDCl_3$): 6.16 (t, 1H, py); 6.85 (d, 1H, py, J = 8.0 Hz); 7.10−7.45 (m, 30H, phenyl); 7.40 (t, 1H, py); and 7.54 (d, 1H, py, J = 8.0 Hz). IR (cm^{-1}, KBr disk): 3030 (m, ν_{C-H}), 1480, 1455,1435,1380 (s, $\nu_{C=C}$), 750, and 700 (s, PPh_3).

[^{99}Tc(HYPY)(tricine)(PPh_3)]. The filtrate above was allowed to evaporate slowly to give a reddish brown solid, which was collected by filtration, washed with a small amount of acetone, and dried in the air. The yield was 5 mg. Anal. Calcd (found) for $C_{29}H_{30}N_4O_5PTc$·1.5H_2O: C, 51.84 (51.94); H, 4.95 (4.52); N, 8.35 (8.22). FAB-MS: m/z = 645 for $[C_{29}H_{31}N_4O_5PTc]^+$. ^{1}H NMR ($CDCl_3$): 2.56 (dd, 1H, CH_2COO); 2.83 (dd, 1H, NH); 3.10 (bs, 1H, OH); 3.40 (d, 1H, CH_2-COO, J = 17.4 Hz), 3.70 (m, 2H, CH_2O); 3.79 (bs, 1H, OH); 3.93 (m, 2H, CH_2O); 4.84 (q, 2H, CH_2O); 6.56 (t, 1H, py); 7.28 (d, 1H, py, J = 5.5 Hz); 7.10−7.30 (m, 15H, phenyl); 7.42 (d, 1H, py, J = 8.2 Hz); and 7.66 (t, 1H, py). ^{13}C NMR ($CDCl_3$, atom labels are shown in Figure 8): 45.19 (C2), 61.57 (C6), 65.66 (C4), 67.14 (C3), 78.26 (C5), 116.85 (C10), 119.28 (C8), 128.10 (C15), 129.80 (C14), 130.65 (C12, J_{P-C} = 43.8 Hz), 132.97 (C13), 137.71 (C9), 145.70 (C11), 153.47 (C7), and 183.30 (C1). IR (cm^{-1}, KBr disk): 3700−3100 (bs, ν_{N-H} and ν_{O-H}), 3056 (m, ν_{C-H}), 1647 (s, $\nu_{C=O}$), 1452 and 1435 (m, $\nu_{C=C}$).

Synthesis of ^{99m}Tc Complexes. One-Step Synthesis Using Stannous Chloride. To a 10 mL vial were added 0.5 mL of $Na^{99m}TcO_4$ solution (100 mCi/mL in saline), 0.2−0.4 mL of tricine solution (100 mg/mL in H_2O), 0.2 mL of HYNICtide solution (50 μg/mL in H_2O), 0.1 mL of phosphine coligand solution (10 mg/mL in H_2O), and 25 μL of $SnCl_2$·2H_2O solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 100 °C for 10 min. After cooling at room temperature for 10 min, the reaction mixture was analyzed by radio-HPLC.

One-Step Synthesis without Stannous Chloride. To a 10 mL vial were added 0.5 mL of $Na^{99m}TcO_4$ solution (100 mCi/mL in saline), 0.2−0.4 mL of tricine solution (100 mg/mL in H_2O), 0.2 mL of HYNICtide solution (50 μg/mL in H_2O), and 0.3 mL of phosphine coligand solution (20 mg/mL in H_2O). The reaction mixture was heated at 100 °C for 10 min. After cooling at room temperature for 10 min, the reaction mixture was analyzed by radio-HPLC. The resulting complexes, RP444, RP445, and RP446, prepared by this method were found by HPLC to be identical to those prepared using stannous formulation.

[^{99m}Tc(HYPY)(tricine)(PPh_3)]. To a clean 10 mL vial was added 0.3 mL of tricine solution (100 mg/mL in H_2O), followed by 0.1 mL of 2-hydrazinopyridine (HYPY) solution (100 μg/mL in H_2O), 0.3 mL of $Na^{99m}TcO_4$ solution (100 mCi/mL in saline), 1.0 mL of triphenylphosphine (PPh_3) solution (2 mg/mL in absolute ethanol), and 20 μL of $SnCl_2$·2H_2O solution (1.0 mg/mL in 0.1 N HCl). The reaction mixture was heated at 50 °C for 30 min and was then analyzed by radio-HPLC (method 1).

HPLC Concordance Experiments. The HPLC concordance experiments were performed for RP444, RP445, and RP446 along with their HPLC-purified ^{99}Tc analogues. The same HPLC method was used in sequence for each pair. The HPLC methods used for these experiments are method 2 for RP444/[^{99}Tc]RP444, method 3 for RP445/[^{99}Tc]-RP445, and method 4 for RP446/[^{99}Tc]RP446.

Chirality Experiment. To a 10 mL vial was added 0.2 mL of tricine solution (100 mg/mL in 25 mM succinate buffer, pH = 5.0), followed by 0.2 mL of TPPTS solution (30 mg/mL in 25 mM succinate buffer, pH = 5.0), 0.2 mL of HYNIC-BA solution (100 μg/mL in 25 mM succinate buffer, pH = 5.0), 0.2 mL of HYNIC-MBA solution (100 μg/mL in 25 mM succinate buffer, pH = 5.0), and 0.2 mL of $Na^{99m}TcO_4$ solution (250 mCi/mL in saline). The reaction mixture was heated at 100 °C for 10 min and was then analyzed by HPLC (method 6).

Solution Stability of HPLC-Purified RP444. RP444 was prepared and purified by HPLC using method 3. Volatiles from the collected fraction were removed under reduced pressure. The residue was diluted with water to a concentration of 5 mCi/mL. The appropriate amount of competing ligands (cysteine methyl ester hydrochloride, histidine methyl ester hydrochloride, and TPPDS) was added to give a concentration of 1.0 mg/mL. Since the HPLC mobile phase contains succinate buffer (pH = 5.0), no additional buffering agent was used for these solutions. The resulting solutions were kept at appropriate temperature (room temperature, 50 or 80 °C) and were analyzed by performing six injections over 6 h.

Solution Stability of Two Isomers of RP444. The two isomeric forms of RP444 were separated by collecting the two radiometric peaks. Volatiles in the collected fractions were removed, and to the residue of each fraction was added TPPTS (1 mg/mL) in water to give a concentration of 3−4 mCi/mL. The resulting solutions were kept in an 80 °C water bath. Samples of these solutions were analyzed using HPLC (method 2) at 2 h intervals over 6 h.

Inhibition of [^{125}I]Fibrinogen Binding to Platelets Assay. The inhibition of binding of [^{125}I]fibrinogen to platelets was performed as described in the literature[14] with some modifications. Canine arterial whole blood was collected into citrate-containing syringes (sodium citrate final concentration 0.38 wt %, Ricca Chemical Co., Arlington, TX). Platelets were isolated and passed through a gel-filtration column containing Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden), and the aliquot was adjusted to achieve a final concentration of 0.1−0.2 million platelets/μL with Tyrode's buffer. This assay was performed in a 96-well microtiter plate, and the reagents (expressed as final concentration) were added in the following order: calcium chloride (2 mM, BioData Corp., Horsham, PA), thrombin (0.1 unit/mL, Sigma), platelets (0.1−0.2 million platelets/μL). After incubating for 2 min at room temperature, hirudin (0.5 unit/mL, Sigma), the test agent ([^{99}Tc]-RP444 or its two isolated isomers), and the fibrinogen mixture ([^{125}I]-fibrinogen 3 μCi/mL and unlabeled fibrinogen 0.83 mg/mL) were added. The microtiter plate was incubated at room temperature for 15 min followed by centrifugation at 3000 rpm for 10 min (model RT6000B rotor H1000B, Sorvall) and the supernatant discarded. The rate of [^{125}I]-fibrinogen incorporation was determined via counting each well on a gamma counter (Model C5003, Packhard Instruments Co.). A concentration−response curve to fibrinogen, determination of nonspecific binding (fibrinogen concentration = 4 mg/mL), and the unknown compound were studied in each assay. All concentrations of each compound tested were run in triplicate. Percent inhibition of [^{125}I]-fibrinogen binding to activated platelets was calculated by dividing the specific binding (total binding − nonspecific binding) obtained in the presence of inhibitors by that obtained in the absence of the inhibitors. The percent nonspecific binding on average was less than 20%. The IC_{50} value was calculated by fitting the percent inhibition values to a regression line.

(14) Marguerie, G. A.; Plow, E. F.; Edgington, T. S. *J. Biol. Chem.* **1979**, *254*, 5357.

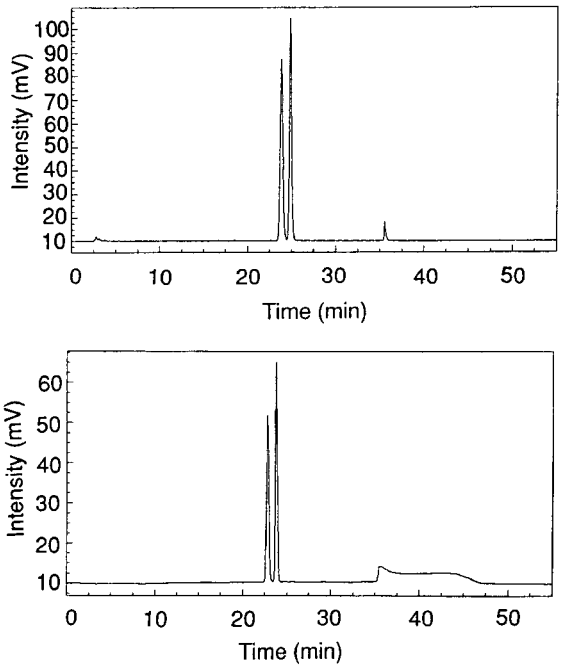


Figure 1. HPLC concordance for RP444 (top) and [^{99}Tc]RP444 (bottom).

Results and Discussion

^{99m}Tc Complexes of HYNICtide. RP444, RP445, and RP446 were synthesized by reacting HYNICtide with $Na^{99m}TcO_4$ in the presence of TPPTS, excess tricine, and stannous chloride at 80 °C for 30 min. They were also prepared using a non-stannous formulation since phosphine coligands can act as reducing agents for [^{99m}Tc]pertechnetate. The non-stannous formulation usually required a higher concentration of the phosphine coligand in the reaction mixture. Radiochemical yield was routinely ≥90%.

^{99}Tc Complexes of HYNICtide. We prepared complexes, [^{99}Tc]RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446, by reacting the HYNICtide with $[NH_4][^{99}TcO_4]$ or $[n\text{-}Bu_4N][^{99}TcOCl_4]$ in the presence of excess tricine and the corresponding phosphine coligand. The yields of these ternary ligand technetium complexes using $[n\text{-}Bu_4N][^{99}TcOCl_4]$ as the starting material were usually very low (≤10%) at the 1−5 mg scale. The yield was much better (40−45% by UV/visible at λ = 340 nm) using $[NH_4][^{99}TcO_4]$ as the starting material. These complexes were isolated from the reaction mixture by two HPLC purifications and were desalted using H_2O/acetonitrile as the mobile phase. After HPLC purification, the ^{99}Tc complexes were obtained only in 100 μg quantities, which is not enough for extensive NMR or other characterizations. For RP444, we had to make several preparations to have enough material for ^{1}H and ^{31}P NMR studies.

HPLC Concordance. To prove that the same complexes were prepared on both the tracer and macroscopic levels, the HPLC concordance experiments were performed for [^{99}Tc]-RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446. Figure 1 shows the HPLC chromatograms for RP444 (top) and [^{99}Tc]RP444 (bottom). The HPLC chromatograms for RP445 and RP446 along with those of their corresponding ^{99}Tc analogues are included in the Supporting Information (Figures SI and SII). It is clear that the compounds isolated at the ^{99}Tc level are identical to those prepared at the tracer (^{99m}Tc) level.

FAB Mass Spectral Data. High-resolution FAB mass spectra of [^{99}Tc]RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446 were obtained using a mixture of thioglycerol and water as the matrix in a positive ion mode. The data are summarized in Table 1. The low-resolution FAB mass spectrum of [^{99}Tc]RP444 is shown in Figure 2, and those of [^{99}Tc]RP445 and [^{99}Tc]RP446 are shown in Figures SIII and SIV, respectively. In general, all three complexes show their expected (M + 1) molecular ions with *m/z* = 1679 for [^{99}Tc]RP444, *m/z* = 1577 for [^{99}Tc]RP445, and *m/z* = 1475 for [^{99}Tc]RP446. The mass spectral data supports the proposed structure (Chart 1) and is completely consistent with the 1:1:1:1 composition for Tc:HYNICtide:tricine:L (L = TPPTS, TPPDS, and TPPMS) in these complexes as determined via a series of mixed-ligand experiments on the tracer (^{99m}Tc) level.[8]

In addition to their molecular ions, these complexes also exhibit M − 22*n* (*n* = 1, 2, or 3 depending on the number of sulfonato groups on the phosphine coligand) peaks arising from substitution of a proton for each sodium ion, as well as the M + 22*n* (*n* = 1 or 2) peaks. The observation of peaks at M + 22 and M + 44 for all three complexes is significant and suggests that two other protons in these molecules are exchangeable with sodium. These two protons are probably from the carboxylic group and the coordinated hydroxy group of the tricine coligand. The model compex [^{99}Tc(HYPY)(tricine)(PPh_3)] contains no substituents on the pyridine ring and showed only one (M + Na) peak at *m/z* = 667 with very low intensity in addition to the expected molecular ion (M + H) at *m/z* = 645. When trifluoroacetic acid (TFA) is added into the matrix, the peaks at M + 22 and M + 44 almost disappear. For example, the predominant peak for [^{99}Tc]RP445 is *m/z* = 1534 corresponding to the ion $[M - 2Na + 3H]^+$ while the main peak for [^{99}Tc]-RP446 is *m/z* = 1453 due to $[M - Na + H]^+$ (Figure SV).

NMR Data. The 600 MHz ^{1}H NMR spectrum of [^{99}Tc]-RP444 (Figure 3) was obtained using D_2O as the solvent. The resonance signals from exchangeable protons (amide NH, hydroxy OH, amine NH, and guanine NH/NH_2) are not seen. Due to the complexity of these spectra, specific assignment is very difficult even with the help of the COSY spectra. Integration of the aromatic region (7.00−8.50 ppm) shows 18 protons from TPPTS, pyridine heterocycle, and the benzene ring of the Mamb moiety while integration of the aliphatic region shows a total of 40 protons from the coordinated tricine, the cyclic peptide backbone, and the caproic acid linker. These data are completely consistent with the proposed structure (Chart 1) and the 1:1:1:1 ratio for Tc:HYNICtide:tricine:TPPTS as demonstrated by FAB mass spectroscopy. The ^{31}P NMR spectra of [^{99}Tc]RP444 showed a broad singlet at 42.2 ppm. Upon coordination to the Tc center, the ^{31}P resonance signal of TPPTS undergoes a downfiled shift of ∼47.5 ppm.

The presence of two isomers is obvious. For example, the ^{1}H NMR spectrum of HYNICtide shows a pair of doublets at 0.92 and 1.10 ppm due to the two methyl groups of valine amino acid, and a singlet at 3.00 ppm due to the methyl group of the methyl-arginine amino acid. For [^{99}Tc]RP444 (Figure 3), however, the ^{1}H NMR spectrum shows two pairs of doublets at 0.95−1.15 ppm from two valine methyl groups, and a pair of singlets at 3.17 ppm from the methyl group of the methyl-arginine amino acid.

Chirality Experiment. For quite a long time, we have been puzzled by the presence of two radiometric peaks in the HPLC chromatograms of RP444, RP445, and RP446. The area ratio of the two peaks is always close to 1:1. Several possible explanations can be envisaged for the observation of two peaks, including the resolution of diastereomers resulting from the chiral HYNICtide and the chiral technetium chelate, and

Table 1. High-Resolution FAB Mass Spectral Data for [^{99}Tc]RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446

compound	formula $[M + H]^+$	found (*m*/*z*)	DEV (ppm)	mass range	TIC	base int.
[^{99}Tc]RP444	$C_{62}H_{76}N_{14}Na_3O_{23}PS_3Tc$	1 679.280 53	−3.50	1679−1680	82 849	101 912
[^{99}Tc]RP445	$C_{62}H_{77}N_{14}Na_2O_{20}PS_2Tc$	1 577.358 74	3.88	1577−1578	296 808	344 973
[^{99}Tc]RP446	$C_{62}H_{78}N_{14}NaO_{17}PSTc$	1 475.410 14	0.89	1475−1476	1 149 034	1 544 285

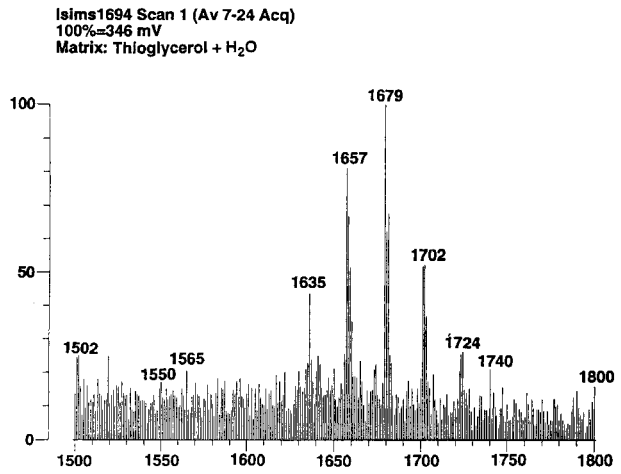


Figure 2. FAB mass spectrum of [^{99}Tc]RP444 (matrix: thioglycerol + H_2O).

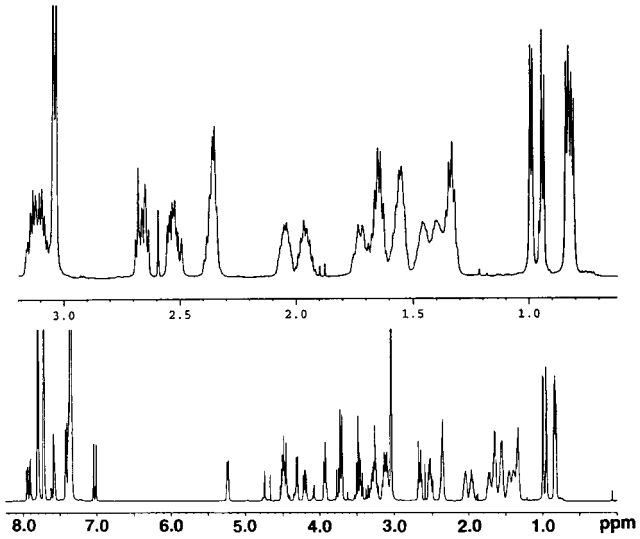


Figure 3. ^{1}H NMR spectrum of [^{99}Tc]RP444 in D_2O.

different geometric orientations of the diazenido ligand due to restricted rotation around the Tc═N and N═N bonds.

To understand the presence of these two isomeric forms, a chirality experiment was designed and performed at the tracer level (^{99m}Tc). In this experiment two closely related HYNIC derivatives, *N*-benzyl-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-BA) and *N*-((*R*)-(+)-α-methylbenzyl)-6-(2-sulfobenzaldehydehydrazono)nicotinamide (HYNIC-MBA) were used in the same reaction mixture. HYNIC-BA has no chiral center while HYNIC-MBA contains a chiral center. The ternary ligand technetium complexes [^{99m}Tc(HYNIC-BA)-(tricine)(TPPTS)] and [^{99m}Tc(HYNIC-MBA)(tricine)(TPPTS)] were prepared by reacting Na[$^{99m}TcO_4$], tricine, TPPTS, and HYNIC-BA/HYNIC-MBA. The resulting solution was analyzed by HPLC (method 6).

If the two isomeric forms of RP444 were caused by different geometric orientations of the pyridine ring in the diazenido ligand as a result of the restricted rotation around the Tc═N and N═N bonds, both HYNIC-BA and HYNIC-MBA would

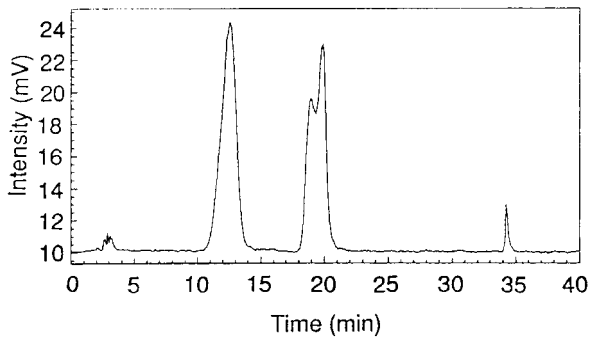


Figure 4. HPLC chromatogram of a mixture of [^{99m}Tc(HYNIC-BA)-(tricine)(TPPTS)]) and [^{99m}Tc(HYNIC-MBA)(tricine)(TPPTS)].

form ternary ligand technetium complexes with HPLC characteristics similar to those of RP444. The HPLC chromatogram of the reaction mixture above would show two pairs of radiometric peaks: one pair from the complex [^{99m}Tc(HYNIC-BA)(tricine)(TPPTS)] and the other pair from the complex [^{99m}Tc(HYNIC-MBA)(tricine)(TPPTS)]. On the contrary, the radio-HPLC chromatogram (Figure 4) of the resulting mixture shows a single peak at 12.5 min due to the complex [^{99m}Tc-(HYNIC-BA)(tricine)(TPPTS)], and a pair of peaks at 19.8 and 20.4 min due to [^{99m}Tc(HYNIC-MBA)(tricine)(TPPTS)]. This finding was confirmed by several other reversed-phase HPLC methods and provides strong evidence that the presence of the two radiometric peaks for the complex [^{99m}Tc(HYNIC-MBA)-(tricine)(TPPTS)] is due to the existence of the chiral center in HYNIC-MBA.

Solution Stability of Technetium Complexes of HYNIC-tide. Previously, it was found that RP444 is stable for more than 12 h both in the kit matrix and in dilute solution.[8] Animal and early clinical studies showed that RP444 is renally excreted without any metabolism.[9] In this study, we found that the HPLC-purified [^{99}Tc]RP444 remains stable in aqueous solution for more than 6 months without any decomposition, and that the isolated two isomers of RP444 undergo no interconversion in aqueous solution at room temperature during that period of time. It is amazing that three different ligands (HYNICtide, tricine, and TPPTS) combine with Tc and form a technetium complex with only two detectable isomers and with extremely high solution stability.

We also studied the solution stability of RP444 in the presence of three different challenge ligands: TPPDS, histidine methyl ester (His-OMe), and cysteine methyl ester (Cys-OMe). We used RP444 instead of [^{99}Tc]RP444 for two reasons. First, it is easier to achieve a large excess of challenge ligand versus RP444 since the total technetium complex concentration is only $\sim 10^{-8}$ M at the tracer level. Second, the radiometric detector can detect any ^{99m}Tc species, which may be produced from the reaction of RP444 with a large excess of challenge ligand, at concentrations as low as $\sim 10^{-10}$ M while the detection limit of the UV/visible detector is generally much higher ($\sim 10^{-6}$ M).

RP444 was first synthesized and purified by HPLC (method 2). Volatiles in the collected fraction were removed under reduced pressure, and residues were redissoved in water to give

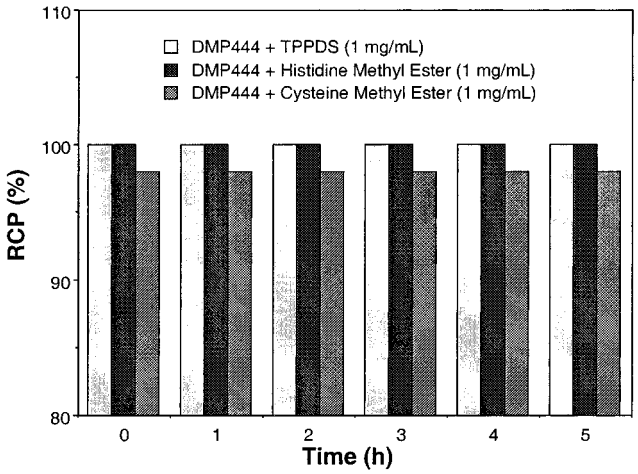


Figure 5. RCP versus time for RP444 in the presence of excess TPPDS, histidine methyl ester, and cysteine methyl ester.

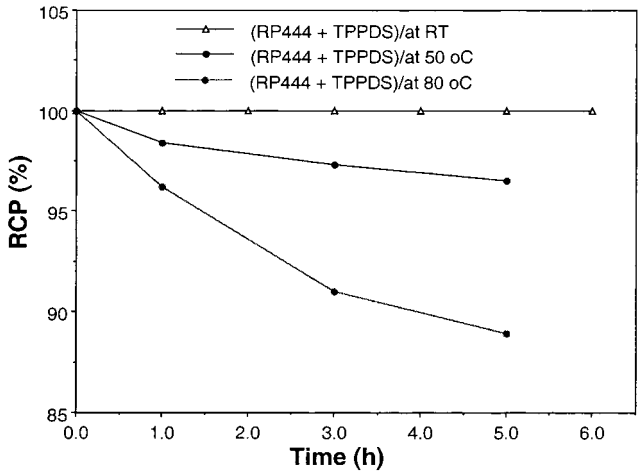


Figure 6. RCP versus time for RP444 in the presence of excess TPPDS at (top to bottom) room temperature, 50 °C, and 80 °C.

a concentration of 5 mCi/mL ($\sim 3.5 \times 10^{-8}$ M). The solution was divided into three portions, which were placed into three lead-shielded vials. TPPDS, histidine methyl ester, and cysteine methyl ester were added to these vials separately to give a concentration of 1 mg/mL (TPPDS, $\sim 1.5 \times 10^{-3}$ M; histidine methyl ester, $\sim 4 \times 10^{-3}$ M; cysteine methyl ester, $\sim 5 \times 10^{-3}$ M). These three solutions were kept at room temperature and monitored by radio-HPLC (method 2). Figure 5 shows a histogram for the stability of RP444 in the presence of excess TPPDS, histidine methyl ester, and cysteine methyl ester. To our surprise, there is no ligand exchange between TPPTS in RP444 and either of the three challenge ligands at room temperature over 6 h even though the challenge ligand is in large excess.

The results from the ligand (cysteine methyl ester and histidine methyl ester) challenge experiments have important implications. Previously, we found that RP444 has a prolonged blood retention ($t_{1/2} = 90$ min).[9] It was also found that ~30% of the injected dose for RP444 binds to circulating platelets and proteins. There are several possible ways that RP444 can bind to those blood components: chemical reaction of RP444 with amino acid residues on proteins, RGD sequence binding to the IIb/IIIa receptors on unstimulated platelets, and nonspecific binding via either hydrophilic or hydrophobic interactions with proteins. Since most proteins are rich in histidine and cysteine amino acids, we decided to use excess histidine methyl ester and cysteine methyl ester to challenge TPPTS in RP444. The high stability of RP444 in the presence of excess histidine methyl ester and cysteine methyl ester strongly suggests that it binds to blood proteins not by any chemical reaction such as ligand displacement of the monodentate TPPTS coligand.

The fact that RP444 remains intact in the presence of ≥30000-fold excess of TPPDS, which is a stronger coligand than TPPTS,[8] is significant. This suggests that the ligand exchange reaction is not dependent on the concentration of the entering ligand. It also suggests that RP444 has an octahedral coordination geometry around the Tc with a monodentate HYNICtide, a TPPTS, and a tetradentate tricine coligand. Five- or seven-coordinated complexes are often kinetically labile and undergo facile associative-addition or dissociative-elimination reactions.

We also studied the solution stability of RP444 in the presence of excess TPPDS at elevated temperatures (50 and 80 °C). Figure 6 shows the plot of RCP versus time for RP444 in the presence of excess TPPDS at room temperature, 50 °C, and 80 °C. It was found that there is a slight drop (3.5%) in RCP over 6 h at 50 °C. At 80 °C, however, the RCP drops ~11% over 5 h. Part of RP444 is converted to RP445, and part of RP444 decomposes to form [^{99m}Tc]pertechnetate at 3.1 min and some very hydrophilic species at the void volume (2.3 min). The high resistance of RP444 to ligand exchange suggests that dissociation of the Tc−P (TPPTS) bond is the rate-limiting step, and the high solution stability of RP444 is most likely due to its kinetic inertness.

We isolated the two isomeric forms of RP444 by collecting the two radiometric peaks separately. Volatiles in the collected fractions were removed, and to the residue of each fraction was added TPPTS (1 mg/mL) in water to give a concentration of 3−4 mCi/mL. The resulting solutions were kept in an 80 °C water bath. Samples of these solutions were analyzed at 2 h intervals over 6 h. It was found that there is no interconversion between the two isomeric forms although some decomposition was observed at 80 °C. This suggests that both isomers of RP444 are kinetically inert with respect to ligand exchange, and that when TPPTS in RP444 is dissociated the five-coordinate intermediate is not stable without a "soft" phosphine coligand present.

In the past decade, the ligand design for ^{99m}Tc radiopharmaceuticals has been focused on preparing polydentate chelators such as N_3S triamidethiols,[15−17] N_2S_2 diamidedithiols,[17,18−21] N_2S_2 diaminedithiols,[22−30] and N_2S_2 monoaminemonoamide-

(15) Bormans, G.; Cleynhens, B.; Adriaens, P.; De Roo, M.; Verbruggen, A. *J. Labelled Compd. and Radiopharm.* **1993**, *33*, 1065.
(16) Grummon, G.; Rajagopalan, R.; Palenik, G. J.; Koziol, A. E.; Nosco, D. L. *Inorg. Chem.* **1995**, *34*, 1764.
(17) Liu, S.; Edwards, D. S. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 4*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; SGEditorali: Padova, 1995, pp 383−393.
(18) Davison, A.; Jones, A. G.; Orvig, C.; Sohn, M. *Inorg. Chem.* **1981**, *20*, 1629.
(19) Rao, T. N.; Adhikesavalu, D.; Camerman, A.; Fritzberg, A. R. *J. Am. Chem. Soc.* **1990**, *112*, 5798.
(20) Fritzberg, A. R.; Abrams, P. G.; Beaumier, P. L.; Kasina, S.; Morgan, A. C.; Rao, T. N.; Reno, J. M.; Sanderson, J. A.; Srinivasan, A.; Wilbur, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4025.
(21) Kasina, S.; Rao, T. N.; Srinivasan, A.; Sanderson, J. A.; Fitzner, J. N.; Reno, J. M.; Beaumier, P. L.; Fritzberg, A. R. *J. Nucl. Med.* **1988**, *32*, 1445.
(22) Edwards, D. S.; Cheesman, E. H.; Watson, M. W.; Maheu, L. J.; Nguyen, S. A.; Dimitre, L.; Nason, T.; Watson, A. D.; Walovitch, R. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 3*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; Cortina International: Verona, 1990; pp 431−444.
(23) Kung, H. F.; Molnar, M.; Billings, J.; Blau, M. *J. Nucl. Med.* **1984**, *25*, 326.

Table 2. IC_{50} (nM) of Ternary Ligand ^{99}Tc Complexes against Fibrinogen Binding to Activated Canine Platelets

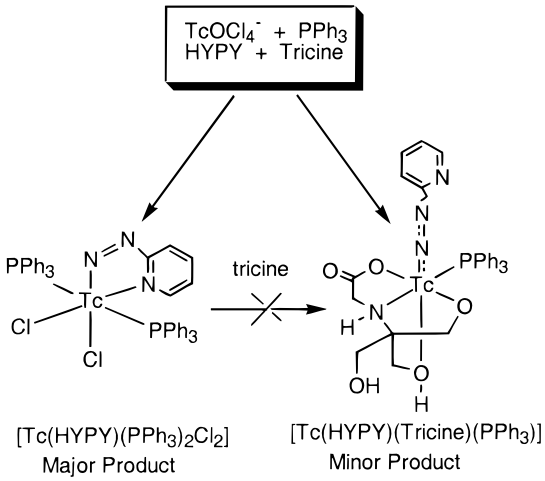
compound	IC_{50} (nM)[a]	ref
DMP757	6 ± 2	39
XV120	6 ± 2 ($n = 4$)	13
[^{99}Tc]RP444	13 ± 2 ($n = 3$)	this work
isomer 1 (22 min peak)	14 ± 2 ($n = 2$)	this work
isomer 2 (24 min peak)	12 ± 1 ($n = 2$)	this work
[^{99}Tc]RP445	11 ± 2 ($n = 2$)	this work
[^{99}Tc]RP446	13 ± 2 ($n = 2$)	this work

[a] The IC_{50} value is an average of several determinations; each determination was performed in triplicate.

dithiols.[31−34] These chelators form technetium complexes with a 1:1 Tc:ligand ratio. The technetium complexes are thermodynamically stable and kinetically inert probably due to the chelate effect. Some of them have been successfully used as bifunctional chelators for the ^{99m}Tc-labeling of proteins[20,21,30] and peptides.[17] Tridentate ligands containing SNS donors were used in the synthesis of brain-imaging agents[35,36] and for the ^{99m}Tc-labeling of dopamine transporters.[37,38] The combination of the dianionic tridentate SNS ligand with a monodentate thiolate produces a binary ligand system that can bind to the Tc═O core and form neutral square-pyramidal technetium complexes. The ternary ligand system described in this study contains three different ligands: a bifunctional coupling group (HYNIC), a monodentate phosphine, and the tetradentate tricine coligand. This represents the first example of a ternary ligand system used for the ^{99m}Tc-labeling of peptides or other biologically active molecules.

Biological Properties of ^{99}Tc Complexes. Complexes [^{99}Tc]-RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446 were prepared, purified by HPLC, isolated as a mixture of two isomeric forms, and evaluated in vitro against fibrinogen binding to activated canine platelets (Table 2). The two isomers of [^{99}Tc]RP444 were also evaluated in the same in vitro assay. It was found that both isomers have the same binding affinity. Compared to the free HYNICtide ($IC_{50} = 6 \pm 2$ and 8.1 ± 1.7 nM against fibrinogen binding to the activated human and canine platelets, respectively),[13,39] [^{99}Tc]RP444, [^{99}Tc]RP445, and [^{99}Tc]RP446 remain high-affinity GPIIb/IIIa receptor antagonists. The ^{99m}Tc-labeling did not significantly decrease the receptor binding affinity.

Chart 2. Synthesis of [^{99}Tc(HYPY)(tricine)(PPh_3)]



Technetium Complexes of HYPY. We have had difficulties in isolating sufficient quantities of ternary ligand ^{99}Tc complexes of the cyclic HYNICtide for rigorous physical and chemical characterizations. The HPLC purifications are usually tedious and time-consuming. The recovery of samples is very low. To explore the coordination chemistry of this new ternary ligand system, we used HYPY as the model for HYNICtide, and triphenylphosphine (PPh_3) for TPPTS.

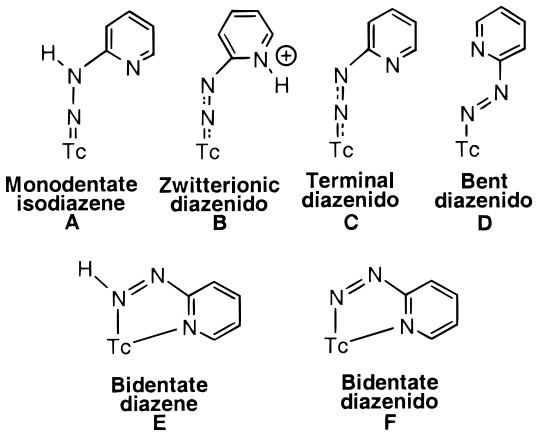
We used both [n-Bu_4N][$^{99}TcOCl_4$] and [NH_4][$^{99}TcO_4$] as the starting materials and reacted them with 2−5 equiv of HYPY·2HCl, 2−3 equival of PPh_3, and a large excess (≥10 equiv) of tricine. Once again, we found that the chemistry at the macroscopic level (25−250 mg) is very different from that at the tracer level (10−100 ng for 10−100 mCi of activity). At the tracer level, the yield for the ternary ligand complex [^{99m}Tc-(HYPY)(tricine)(PPh_3)] was high (≥85%). At the macroscopic level, however, the yield of [^{99}Tc(HYPY)(tricine)(PPh_3)] was less than 10%, and the major product of the reaction is [^{99}Tc-(HYPY)(PPh_3)$_2Cl_2$] (Chart 2). The hydrophobicity of the two PPh_3 coligands no doubt facilitates the precipitation of the complex [^{99}Tc(HYPY)(PPh_3)$_2Cl_2$]. Both [^{99}Tc(HYPY)(tricine)-(PPh_3)] and [^{99}Tc(HYPY)(PPh_3)$_2Cl_2$] have been characterized by HPLC, IR, NMR (^{1}H and ^{13}C), FAB-MS, and elemental analysis. Unfortunately, we were unable to grow single crystals for an X-ray diffraction study, despite many attempts using a variety of solvent combinations.

To confirm the neutrality of [Tc(HYPY)(tricine)(PPh_3)], we reacted it with silver(I) triflate in a mixture of acetonitrile and methanol. The absence of silver chloride precipitate indicates that there is no labile halide present. Since the ^{1}H NMR spectrum of the complex [Tc(HYPY)(tricine)(PPh_3)] shows no proton resonance signals from tetrabutylammonium cation, the complex is not anionic either. Therefore, the complex [Tc-(HYPY)(tricine)(PPh_3)] is neutral.

[Tc(HYPY)(tricine)(PPh_3)] shows IR bands at 1640 cm^{-1} from the coordinated carboxylate. Upon coordination to the

(24) Kung, H. F.; Guo, Y. Z.; Yu, C. C.; Billings, J.; Subramanyam, V.; Calabrese, J. C. *J. Med. Chem.* **1989**, *32*, 433−437.
(25) Oya, S.; Kung, M. P.; Frederick, D.; Kung, H. F. *Nucl. Med. Biol.* **1995**, *22*, 749.
(26) Francesconi, L. C.; Graczyk, G.; Wehrli, S.; Shaikh, S. N.; McClinton, D.; Liu, S.; Zubieta, J.; Kung, H. F. *Inorg. Chem.* **1993**, *32*, 3114.
(27) Lever, S. Z.; Sun, S.-Y.; Scheffel, U. A.; Kaltovich, F. A.; Baidoo, K. E.; Goldfarb, H.; Wagner, Jr., H. N. *J. Pharm. Sci.* **1994**, *84*, 802.
(28) Baidoo, K. E.; Lever, S. Z. *Bioconjugate Chem.* **1990**, *1*, 132.
(29) Eisenhut, M.; Mibfeldt, M.; Lehmann, W. D.; Karas, M. *J. Labelled Compd. Radiopharm.* **1991**, *29*, 1283.
(30) Eisenhut, M.; Lehmann, W. D.; Becker, W.; Elser, H.; Strittmatter, W.; Baum, R. P.; Valerius, T.; Repp, R.; Deo, Y. *J. Nucl. Med.* **1996**, *37*, 362.
(31) O'Neil, J. P.; Wilson, S. R.; Katzenellenbogen, J. A. *Inorg. Chem.* **1994**, *33*, 319.
(32) Gustavson, L. M.; Rao, T. N.; Jones, D. S.; Fritzberg, A. R. *Tetrahedron Lett.* **1991**, *32*, 5485.
(33) Mahmood, A.; Wolff, J. A.; Davison, A.; Jones, A. J. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 4*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; SGEditorali: Padova, 1995; pp 211−215.
(34) Kung, H. F.; Bradshaw, J. E.; Chumpradit, S.; Zhang, Z. P.; Kung, M. P.; Mu, M.; Frederick, D. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 4*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; SGEditorali: Padova, 1995; pp 293−298.
(35) Spyriounis, D. M.; Pelecanou, M.; Stassinopoulou, C. I.; Raptopoulou, C. P.; Terzis, A.; Chiotellis, E. *Inorg. Chem.* **1995**, *34*, 1077.
(36) Mastrostamatis, S. G.; Papadopoulos, M. S.; Pirmettis, I. C.; Paschali, E.; Varvarigou, A. D.; Stassinopoulou, C. I.; Raptopoulou, C. P.; Terzis, A.; Chiotellis, E. *J. Med. Chem.* **1994**, *37*, 1077.
(37) Meegalla, S.; Plössl, K.; Kung, M.-P.; Stevenson, D. A.; Liable-Sand, L. M.; Rheingold, A. L.; Kung, H. F. *J. Am. Chem. Soc.* **1995**, *117*, 11037.
(38) Meegalla, S.; Plössl, K.; Kung, M.-P.; Chumpradit, S.; Stevenson, D. A.; Frederick, D.; Kung, H. F. *Bioconjugate Chem.* **1996**, *7*, 421.
(39) Jackson, S.; DeGrado, W. F.; Dwivedi, A.; Parthasarathy, A.; Higley, A.; Krywko, J.; Rockwell, A.; Markwalder, J.; Wells, G.; Wexler, R.; Mousa, S.; Harlow, R. *J. Am. Chem. Soc.* **1994**, *116*, 3220.

Chart 3. Several Bonding Modalities of HYPY



metal ion, the C═O stretching frequency undergoes a bathochromic shift (∼100 cm^{-1}). The broad band at 3700−3200 cm^{-1} is probably due to O−H stretches from either uncoordinated hydroxy group of tricine or crystallization H_2O. The bands at 3050−2600 cm^{-1} are due to aromatic C−H and tricine C−H stretches. These data clearly demonstrate the presence of the coordinated triphenylphosphine and tricine ligands in the complex [Tc(HYPY)(tricine)(PPh_3)].

Complexes containing Tc−hydrazido and Tc−diazenido bonds have been reported previously and characterized by X-ray crystallography.[40−48] There are several bonding modes for HYPY (Chart 3). Recently, Davison and co-workers reported a series of technetium(III) and rhenium(III) complexes of HYPY.[40,41] It was found that HYPY has several bonding modalities: neutral bidentate pyridyldiazene,[40,41] neutral monodentate pyridiniumdiazenido,[40] and anionic monodentate pyridyldiazenido.[41] Various technetium and rhenium complexes can be prepared depending upon the starting material and reaction conditions. For example, the reaction of $NH_4[TcO_4]$ with HYPY·2HCl in methanol produces a complex [$TcCl_3$(HN═NC_5H_4N)(N═NC_5H_4NH)]. The reaction of the complex [$ReCl_3$(HN═NC_5H_4N)(N═NC_5H_4NH)] with triphenylphosphine (PPh_3) in the presence of a base such as diisopropylethylamine gives a complex [$ReCl_2(PPh_3)$(N═NC_5H_4N)(NH═NC_5H_4N)], in which the N═NC_5H_4N moiety was found to be an anionic monodentate pyridyldiazenido.[40]

The hydrogen of the Tc−NH═N moiety (**E** in Chart 3) is heavily deshielded by the N═N double bond and the Tc center. It was reported that the resonance signal for that proton appeared at ∼22 ppm in the ^{1}H NMR spectrum of the complex [Tc(NH═NPy)(N═NPy)Cl_3].[40,41] The hydrogen of the Tc═N−NH moiety (**A** in Chart 3) also experiences a deshielding effect from the N═N double bond and the aromatic pyridine ring. In the ^{1}H NMR spectrum of HYPY in $CDCl_3$, the signal due to the proton on the α-N atom of HYPY appears at 6.2 ppm. Upon coordination to the Tc, the resonance signal for this proton should appear further downfield (8−20 ppm).

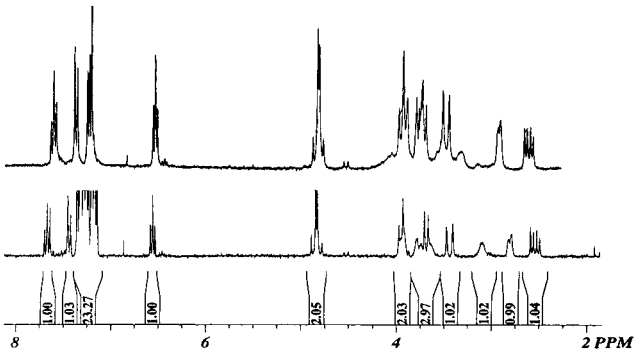


Figure 7. ^{1}H NMR spectra of complexes [^{99}Tc(HYPY)(tricine)(PPh_3-d_5)] (top) and [^{99}Tc(HYPY)(tricine)(PPh_3)] (bottom) in $CDCl_3$.

Figure 7 shows the ^{1}H NMR spectra of [Tc(HYPY)(tricine)(PPh_3)] (top) and [Tc(HYPY)(tricine)(PPh_3-d_5)] (bottom) in $CDCl_3$. The ternary ligand complex [Tc(HYPY)(tricine)(PPh_3-d_5)] was prepared, and its ^{1}H NMR spectrum was compared to that of [Tc(HYPY)(tricine)(PPh_3)] to eliminate the possibility that the proton resonance signal due to either the Tc−NH═N or Tc═N−NH moiety is superimposed with those of triphenylphosphine. It was found that there is no resonance signal in the 8−20 ppm region from either Tc−NH═N or Tc═N−NH. This stongly suggests that HYPY bind to the Tc via a Tc−diazenido bond (**C** in Chart 3). In the range 2.4−5.0 ppm, the spectrum (Figure 7) shows signals due to NH hydrogen and eight distinctive resonance signals from the methylene hydrogens of the coordinated tricine. Integration of all proton signals shows that the complex contains only one HYPY, one tricine, and one triphenylphosphine ligand. This is completely consistent with the proposed structure (Chart 3).

The ^{1}H NMR spectrum of the uncoordinated tricine in D_2O shows a singlet at 3.58 ppm due to three hydroxymethylene groups, and a singlet at 3.33 ppm due to methylene hydrogens adjacent to the carboxylic group. Upon coordination to the Tc center, the technetium chelate becomes a chiral center. It is not surprising that the coordinated tricine shows eight distinctive methylene hydrogen resonance signals in the 2.4−5.0 ppm region. Thus, the coordinated tricine ligand is rigid in solution. This rigidity has been confirmed by variable-temperature ^{1}H NMR studies, which show no signal collapse from room temperature to 50 °C in $CDCl_3$ (Figure 8). No intramolecular exchange of hydroxyl groups was observed. The rigidity of the coordinated tricine suggests that it is most likely tetradentate. Two broad resonance signals are seen at 3.7 and 3.2 ppm (bottom, Figure 7). The broad signal at 3.2 ppm is assigned to the hydrogen of the uncoordinated hydroxyl group while the other one at 3.7 ppm is assigned to the hydrogen of the apical hydroxyl group. In the presence of D_2O, these two hydrogens undergo deuterium exchange.

The ^{1}H NMR spectrum of the complex [Tc(HYPY)(PPh_3)$_2$-Cl_2] in $CDCl_3$ shows a different splitting pattern in the aromatic region (6.0−8.0 ppm), suggesting that HYPY in [Tc(HYPY)(PPh_3)$_2Cl_2$] may have a bonding mode different from that of HYPY in [Tc(HYPY)(tricine)(PPh_3)]. The IR spectrum of the

(40) Nicholson, T.; Cook, J.; Davison, A.; Rose, D. J.; Maresca, K. P.; Zubieta, J. A.; Jones, A. J. *Inorg. Chim. Acta* **1996**, *252*, 421.
(41) Nicholson, T.; Cook, J.; Davison, A.; Rose, D. J.; Maresca, K. P.; Zubieta, J. A.; Jones, A. J. *Inorg. Chim. Acta* **1996**, *252*, 427.
(42) Abrams, M. J.; Larsen, S. K.; Shaikh, S. N.; Zubieta, J. *Inorg. Chim. Acta* **1991**, *185*, 7.
(43) Archer, C. M.; Dilworth, J. R.; Jobanputra, P.; Thompson, R. M.; McPartlin, M.; Povey, D. C.; Smith, G. W.; Kelly, J. D. *Polyhedron* **1990**, *9*, 1497.
(44) Dilworth, J. R.; Jobanputra, P.; Thompson, R. M.; Archer, C. M.; Povey, D. C.; Kelly, J. D.; Hiller, W. *Z. Naturforsch.* **1992**, *46*, 449.
(45) Archer, C. M.; Dilworth, J. R.; Jobanputra, P.; Thompson, R. M.; McPartin, M.; Hiller, W. *J. Chem. Soc., Dalton Trans.* **1993**, 897.
(46) Dilworth, J. R.; Jobanputra, P.; Thompson, R. M.; Povey, D. C.; Archer, C. M.; Kelly, J. D. *J. Chem. Soc., Dalton Trans.* **1994**, 1251.
(47) Nicholson, T.; de Vries, N.; Davison, A.; Jones, A. G. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 3*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; Cortina International: Verona, 1990; pp 95−108.
(48) Cook, J.; Davision, A.; Jones, A. J.; Davis, W. M. In *Technetium and Rhenium in Chemistry and Nuclear Medicine. 3*; Nicolini, M., Banoli, G., Mazzi, U., Eds.; Cortina International: Verona, 1990; pp 65−68.

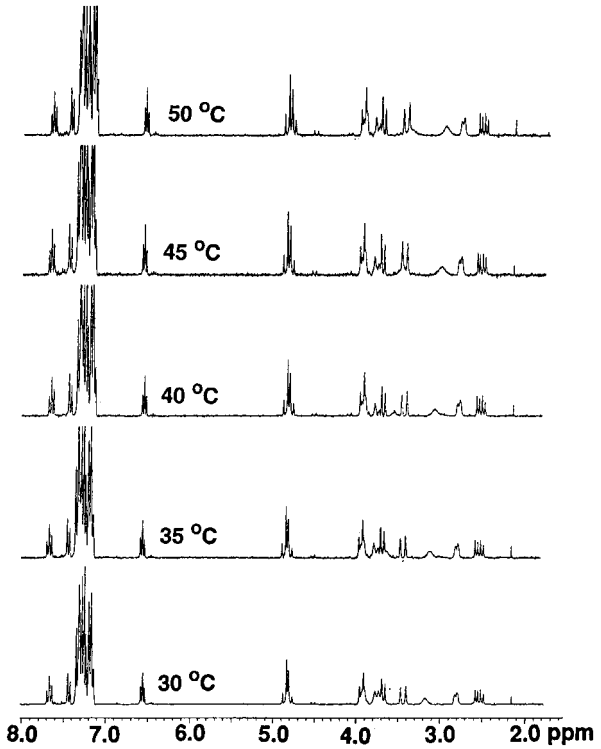


Figure 8. Variable-temperature ^{1}H NMR spectra of [Tc(HYPY)(tricine)(PPh_3)] in $CDCl_3$.

complex [Tc(HYPY)(PPh_3)$_2$$Cl_2$] displays strong absorption at ~700 cm^{-1} from the two coordinated phosphine ligands. We also tried to react [Tc(HYPY)(PPh_3)$_2$$Cl_2$] with excess tricine in the presence of a base and found that it is very difficult to convert into the complex [Tc(HYPY)(tricine)(PPh_3)]. Thus, it is reasonable to assume that HYPY bonds to the Tc atom as a monoanionic bidentate diazenido ligand and the complex [Tc(HYPY)(PPh_3)$_2$$Cl_2$] has an octahedral coordination geometry. The Re(III) complex [Re(HYPY)Cl_2(PPh_3)$_2$] has been characterized by X-ray crystallograpy,[49] and the HYPY ligand was found to be a neutral bidentate pyridyldiazene.

If HYPY bonds to the Tc center as a bidentate ligand (Chart 4), the tricine coligand in the complex [Tc(HYPY)(tricine)(PPh_3)] has to be tridentate to complete the octahedral coordination sphere. Due to the relative geometric arrangement of these three different ligands, there are eight possible isomeric forms: four facial and four meridional (Chart 4). Each of these eight isomers has its enantiomer. The carboxylate group in these different isomers is expected to experience very different chemical and magnetic environments, and the ^{13}C NMR is expected to show at least eight resonance signals from the coordinated carboxylate group. However, the actual ^{13}C spectrum of [Tc(HYPY)(tricine)(PPh_3)] (Figure 9) shows only one resonance signal at 184 ppm due to the coordinated carboxylate. This provides strong evidence for the assumption that tricine in [Tc(HYPY)(tricine)(PPh_3)] is tetradentate and HYPY is a monodentate diazenido ligand. In addition, NMR studies (^{1}H and ^{13}C) of vanadium(V) complexes show that tridentate tricine is quite fluxional with three hydroxyl groups alternating in bonding to the V=O core.[50,51] In contrast, the ^{1}H NMR spectrum of [Tc(HYPY)(tricine)(PPh_3)] shows that the coordinated tricine ligand remains rigid at temperatures higher than 50 °C.

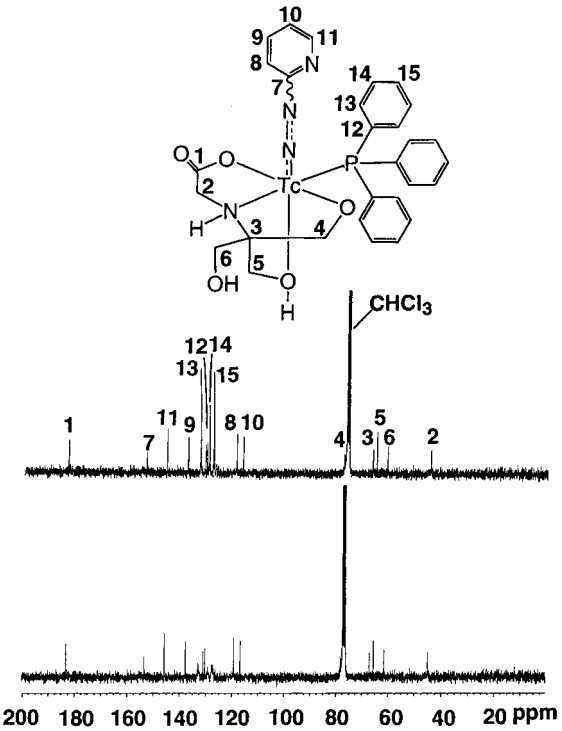
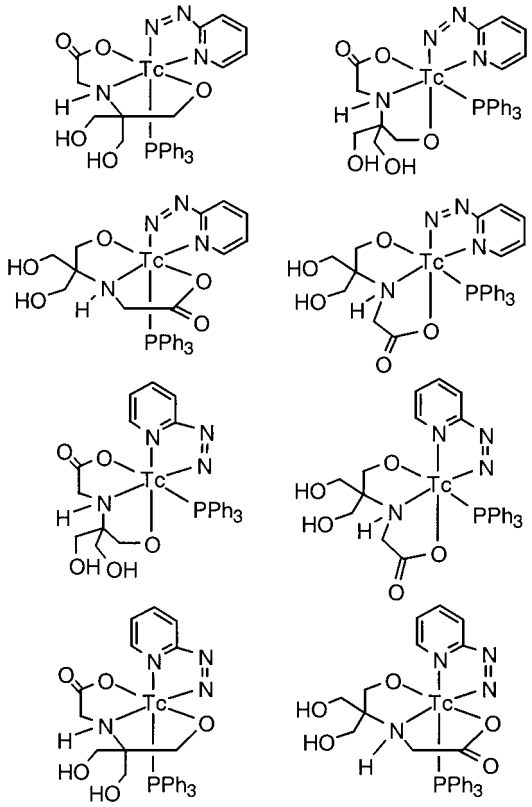


Figure 9. ^{13}C NMR spectra of complexes [^{99}Tc(HYPY)(tricine)(PPh_3)] (top) and [^{99}Tc(HYPY)(tricine)(PPh_3-d_5)] (bottom) in $CDCl_3$.

Chart 4. Eight Isomeric Forms from Bidentate HYPY and Tridentate Tricine in the Ternary Ligand Technetium Complexes



In RP444, RP445, and RP446, the HYNICtide most likely binds to the Tc via a Tc−diazenido bond. The coordination geometry around the Tc is distorted octahedral, and the tricine coligand is tetradentate. Since tricine is prochiral, the technetium chelate is chiral and should be formed in an equal mixture of D

(49) Hirsch-Kuchma, M.; Nicholson, T.; Davison, A.; Davis, W. M.; Jones, A. J. *Inorg. Chem.* **1997**, *36*, 3237.
(50) Crans, D. C.; Ehde, P. M.; Shin, P. K.; Petterson, L. *J. Am. Chem. Soc.* **1991**, *113*, 3728.
(51) Crans, D. C.; Shin, P. K. *J. Am. Chem. Soc.* **1994**, *116*, 1305.

and L enantiomers. These enantiomers in combination with the chiral centers of the HYNICtide result in two diastereomers, DDLL and LDLL. Therefore, it is not surprising that HYNIC-conjugated molecules with one or more chiral centers form ternary ligand [^{99m}Tc]HYNICtide complexes with two radiometric peaks in their radio-HPLC chromatograms as demonstrated in the chirality experiment.

Conclusions

We describe the synthesis, spectroscopic characterization, and biological properties of the corresponding ^{99}Tc analogues of RP444, RP445, and RP446. The HPLC concordance experiments for ^{99m}Tc and ^{99m}Tc analogues show clearly that the same complexes are prepared on the no-carrier-added (^{99m}Tc) and the carrier-added (^{99}Tc) levels. Using a chirality experiment, we demonstrated that the presence of two radiometric peaks in the HPLC chromatograms of RP444, RP445, and RP446 is due to the resolution of diastereomers, which result from the presence of chiral cyclic peptide and the formation of two enantiomers of the technetium chelate. In a ligand challenge experiment, we found that the high solution stability of these ternary ligand [^{99m}Tc]HYNICtide complexes is due to their kinetic inertness. The 1:1:1:1 composition for Tc:HYNICtide:L:tricine (L = TPPTS, TPPDS, and TPPMS) in these ternary ligand [^{99m}Tc]-HYNICtide complexes is confirmed by FAB mass spectral data and is completely consistent with that determined on the tracer (^{99m}Tc) level. In addition, we also determined the IC_{50} values of RP444, RP445, RP446, and the two isomeric forms of RP444 using the platelet IIb/IIIa binding assay. In this assay, both isomeric forms were found to have the same binding affinity ($IC_{50} = 13 \pm 2$ nM).

Complexes [^{99}Tc(HYPY)(PPh_3)$_2Cl_2$] and [^{99}Tc(HYPY)(PPh_3)-(tricine)] were isolated from the reaction of HYPY with [*n*-Bu_4N][$TcOCl_4^-$] in the presence of excess tricine and triphenyphosphine. The complex [^{99}Tc(HYPY)(PPh_3)(tricine)] serves as a model for RP444, RP445, and RP446. It has been characterized by HPLC, spectroscopic (IR, NMR, and FAB-MS) methods, and elemental analysis. The NMR (^{1}H and ^{13}C) data suggests that [^{99}Tc(HYPY)(PPh_3)(tricine)] have an octahedral coordination geometry with a monodentate diazenido HYPY, a tetradentate tricine, and a monodentate phosphine coligand.

Acknowledgment is made to Professor Chris Orvig, Department of Chemistry, The University of British Columbia, for the mass spectra of complexes, [^{99}Tc]RP444, [^{99}Tc]RP445, [^{99}Tc]RP446, [^{99}Tc(HYPY)(PPh_3)$_2Cl_2$], and [^{99}Tc(HYPY)(tricine)(PPh_3)], to John Pietryka for the ^{1}H NMR spectra, and to Dr. M. Rajopadhye, Dr. T. D. Harris, Dr. D. Glowacka, J. P. Bourque, P. R. Damphousse, and K. Yu for the synthesis of HYNICtide.

Supporting Information Available: Radio-HPLC chromatograms (Figures SI and SII) for RP445 and RP446, along with their corresponding ^{99}Tc analogues, and FAB mass spectra (Figures SIII−SV) of [^{99}Tc]RP445 and [^{99}Tc]RP446. This material is available free of charge via the Internet at http://pubs.acs.org.

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